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RESEARCH ARTICLE

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Only IL-1β release is inflammasome-dependent upon ultraviolet B irradiation although IL-18 is also secreted

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Abstract

DNA damage accumulates in aged postmitotic retinal pigment epithelium (RPE) cells, a phenomenon associated with the development of age-related macular degeneration. In this study, we have experimentally induced DNA damage by ultraviolet B (UVB) irradiation in interleukin-1α (IL-1α)-primed ARPE-19 cells and examined inflammasome-mediated signaling. To reveal the mechanisms of inflammasome activation, cells were additionally exposed to high levels of extracellular potassium chloride, n-acetyl-cysteine, or mitochondria-targeted antioxidant MitoTEMPO, prior to UVB irradiation. Levels of interleukin-18 (IL-18) and IL-1β mRNAs were detected with qRT-PCR and secreted amounts of IL-1β, IL-18, and caspase-1 were measured with ELISA. The role of nucleotide-binding domain and leucine-rich repeat pyrin containing protein 3 (NLRP3) in UVB-induced inflammasome activation was verified by using the NLRP3-specific siRNA. Reactive oxygen species (ROS) levels were measured immediately after UVB exposure using the cell-permeant 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) indicator, the levels of cyclobutane pyrimidine dimers were assayed by cell-based ELISA, and the extracellular levels of adenosine triphosphate (ATP) determined using a commercial bioluminescence assay. We found that pro-IL-18 was constitutively expressed by ARPE-19 cells, whereas the expression of pro-IL-1β was inducible by IL-1α priming. UVB induced the release of mature IL-18 and IL-1β but NLRP3 contributed only to the secretion of IL-1β. At the mechanistic level, the release of IL-1β was regulated by K^+ efflux, whereas the secretion of IL-18 was dependent on ROS production. As well as K^+ efflux, the cells released ATP following UVB exposure. Collectively, our data suggest that UVB clearly stimulates the secretion of mature IL-18 as a result of ROS induction, and this response is associated with DNA damage. Moreover, in human

Abbreviations: AMD, age-related macular degeneration; ASC, adaptor protein apoptosis-associated speck-like protein containing a CARD; ATP, adenosine triphosphate; CPD, cyclobutane pyrimidine dimer; ELISA, enzyme-linked immunosorbent assay; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; IL-18, interleukin-18; IL-1α, interleukin-1α; IL-1β, interleukin-1β; KCl, potassium chloride; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; NAC, n-acetyl-cysteine; NLRP3, nucleotide-binding domain and leucine-rich repeat pyrin containing protein 3; qRT-PCR, quantitative real-time polymerase chain reaction; RFU, relative fluorescence unit; ROS, reactive oxygen species; RPE, retinal pigment epithelium; siRNA, small interfering RNA; UVB, ultraviolet B radiation.

RPE cells, K^+ efflux mediates the UVB-activated NLRP3 inflammasome signaling, leading to the processing of IL-1β.

KEYWORDS

DNA damage, NLRP3, IL-1β, IL-18, retinal pigment epithelium

1 | INTRODUCTION

Aging is associated with the presence of elevated amounts of reactive oxygen species (ROS), the accumulation of nonfunctional cellular organelles, as well as persistent DNA damage, such as double-strand breaks and other DNA aberrations.[1,2] Over time, the DNA repair mechanisms tend to become either inadequate or incapable of repairing the increasing amount of DNA damage, resulting in impaired cellular functionality and the appearance of cellular senescence.[1,2] It is believed that the increased DNA damage along with other detrimental cellular events are causative factors in many age-related diseases, such as Alzheimer disease, Parkinson's disease, and age-related macular degeneration (AMD).[1-3]

Nucleotide-binding domain and leucine-rich repeat pyrin containing protein 3 (NLRP3) has been the most widely studied and best-known inflammasome receptor[4]; its main role is to activate the pro-inflammatory cytokines interleukin (IL)-1β and IL-18.[5,6] After NLRP3 has sensed its ligand, ie either a pathogen-associated molecular pattern or a danger-associated molecular pattern, it forms a multiprotein complex with the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) and pro-caspase-1 leading to the auto-activation of caspase-1.[7,8] Active caspase-1 subsequently converts the pro-forms of IL-1β and IL-18 into their mature and bioactive forms, which are subsequently secreted out of the cell[7,8] along with cleaved caspase-1 subunits.[9,10] There are multiple upstream mechanisms through which the inflammasome can be activated, especially K^+ efflux, ROS, and lysosomal rupture are known to contribute to this activation process.[7]

Ultraviolet B (UVB; 290-315 nm) is the most harmful type of ultraviolet radiation to which human tissues are exposed to every day.[11] The photons present in UVB are directly absorbed by DNA; UVB not only can cause direct mutagenic and cytotoxic damage in cells, but it also induces the production of ROS, which indirectly damages DNA.[11-13] Although negligible amounts of UVB reach the retina in adults,[14,15] we have utilized it in our experimental model in order to mimic a DNA-damaged environment in human retinal pigment epithelium (RPE) cells. Although it is known that RPE is vulnerable to DNA damage during aging, it is still unresolved whether or not the NLRP3 inflammasome becomes activated in human RPE cells under these conditions.

2 | MATERIALS AND METHODS

2.1 | Cell stimulations

The human retinal pigment epithelial cell line (ARPE-19; CRL-2302) was purchased from the American Type Culture Collection (ATCC). Cells were cultured in DMEM/F12 (1:1) growth medium (Life Technologies, Paisley, UK) containing 10% of inactivated fetal bovine serum (Hyclone, Logan, UT, USA), 2 mM of L-glutamine (Lonza, Walkersville, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Lonza) at +37°C in humidified conditions with 5% of CO_2. Passages 26-36 were used in the present study.

ARPE-19 cells were seeded onto 6-well plates at a density of 4×10^5 cells per well. Confluent cell layers were washed and replaced with medium containing 100 U/mL penicillin and 100 μg/mL streptomycin without serum and phenol red (DMEM/F12 1:1; Life Technologies). Cells were treated with human recombinant interleukin-1α (IL-1α, 4 ng/mL; R&D Systems, Minneapolis, MN, USA) with or without potassium chloride (KCl, 50 mM; Merck, Darmstadt, Germany) for 48 h at +37°C in humidified conditions with 5% of CO_2. The cell culture medium was replaced with fresh medium and cells were treated with a glutathione precursor, N-acetyl-L-cysteine (NAC, 5 mM; Sigma) or the mitochondrial targeted superoxide dismutase mimetic, MitoTEMPO (200 μM; Enzo, Farmingdale, NY, USA). Both NAC and MitoTEMPO were previously tested on ARPE-19 cells at 5-10 mM[16,17] and at 200-250 μg/mL[17,18] concentrations, respectively. After a one hour incubation at +37°C, the cells were irradiated under a UVB lamp (two TL 20W/12, Philips, Eindhoven, The Netherlands) at an energy level of 2.29 J/cm^2, the optimal level tolerated by these cells. The UVB lamp was calibrated prior to the experiments using a spectroradiometer (Waldman Variocontrol, Germany). After UVB irradiation, the cells were incubated for 1 to 24 h at +37°C in humidified conditions with 5% of CO_2. Cell culture media were collected into microtubes as described previously.[19]

2.2 | Lactate dehydrogenase assay

Cell membrane rupture was measured by the Lactate dehydrogenase (LDH) assay. LDH was detected from freshly

collected cell culture mediums by the commercial CytoTox96 Non-Radioactive Cytotoxicity assay (Promega, Madison, WI, USA) according to the manufacturer's protocol. The colorimetric reaction was measured with a spectrophotometer (ELx808, Biotek Instruments Inc, Winooski, VT, USA) at a wavelength of 490 nm.

2.3 | ELISA measurements

Concentrations of IL-1β (BD Bioscience, San Diego, CA, USA), IL-18 (eBioscience, San Diego, CA, USA), and caspase-1 (p20 subunit; R&D Systems, Minneapolis, MN, USA) were determined from the cell culture medium samples and intracellular NLRP3 (Cusabio, Wuhan, China) from the cell lysates using the ELISA method according to the manufacturers' instructions. Absorbance values were measured by a spectrophotometer at the wavelength of 450 nm with the reference wavelength of 655 nm. The results of intracellular NLRP3 were normalized to total protein concentrations measured by the Bradford method.

2.4 | NLRP3 knockdown

ARPE-19 cells were seeded onto 12-well plates at a density of 1.5×10^5 cells per well for the siRNA experiments. Sub-confluent (60%-80%) cell layers were washed with fresh DMEM/F12 medium without phenol red and antibiotics, and the cells were further treated with Silencer Select NLRP3 siRNA (ID: s41556; Ambion by Life Technologies) with Lipofectamine RNAiMAX Reagent (Invitrogen, Van Allen Way Carlsbad, CA, USA) according to the manufacturer's instructions and as described in our previous study.[20] Silencer Select Negative control siRNA (Ambion) was used as a negative control. The cells were incubated for 24 h, and fresh serum-free medium was placed on the cells with the priming factor IL-1α. Thereafter, the cultures were incubated for 24 h. UVB irradiations and sample collections were performed as mentioned above.

2.5 | Intracellular ROS measurements

ARPE-19 cells were seeded onto 96-well plates at a density of 2×10^4 cells per well in complete growth medium. Confluent cell layers were washed with serum-free medium, and medium containing IL-1α was added prior to incubation for 48 h. The amounts of intracellular ROS were measured in cells treated with 2-10 mM of NAC for 1 hour prior to the addition of cell-permeable 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA, 2.5 μM; Invitrogen, Eugene, OR, USA). Cells were incubated for 30 min at +37°C in humidified conditions with 5% of CO_2 following the complete washes and replacement with fresh phenol red-free medium onto the wells. The levels of hydrolyzed and oxidized 2′,7′-dichlorofluorescein (DCF) were measured with a fluorometer (Cytation 3, Biotek Instruments, Inc Winooski, VT, USA) immediately after UVB exposure using the excitation and emission wavelengths of 488 and 528 nm, respectively. Hydrogen peroxide (1 mM, Sigma) was used as a positive control. Cells without H_2DCFDA treatment provided the background signal, which was subtracted from the results obtained from the study samples.

2.6 | Adenosine triphosphate detection

Adenosine triphosphate (ATP) levels were detected from cell culture medium by a commercial luciferase-based bioluminescence assay (Molecular Probes, Invitrogen). Luminescence was measured according to the manufacturer's protocol in a luminometer (Cytation 3).

2.7 | Quantitative RT-PCR

Based on previous studies,20,21 ARPE-19 cells were exposed to IL-1α (4 ng/mL) for 3 and 6 hours, and then, the cells were collected in ice-cold 1 × DPBS and centrifuged for 1 min at 16 060 *g* at 4°C. Total RNA was isolated from the lysed cells using the commercial NucleoSpin RNA/Protein extraction kit (Macherey-Nagel, Düren, Germany). RNA purity and concentration were measured by Nanodrop (A260/280 nm). cDNA was synthesized from 500 ng of total RNA using the SuperScript III First-Strand synthesis for RT-PCR kit (Invitrogen). Quantitative PCR was performed with the Applied Biosystems 7500 Real time PCR System (Applied Biosystems by Life Technologies Europe BV) according to the protocol from PowerUP SYBR Green master mix (Invitrogen). The following primers were used in qPCR: IL-18, forward 5′-CAGCCTAGAGGTATGGCTGT-3′ and reverse 5′-TCATGTCCTGGGACACTTCTC-3′ (Metabion, Steinkirchen, Germany), IL-1β, forward 5′-AAAAGCTTGGT GATGTCTGG-3′ and reverse 5′-TTTCAACACGCAGGA CAGG-3′ (TAG Copenhagen, Copenhagen, Denmark), and β-actin, forward 5′-GGATGCAGAAGGAGATCACTG-3′ and reverse 5′-CGATCCACACGGAGTACTTG-3′ (TAG Copenhagen). A non-template control was included in every measurement for each gene. The mRNA expressions of IL-1β and IL-18 were normalized to the levels of the housekeeping gene, β-actin. Changes in the mRNA expression were calculated with the DD CT method.

2.8 | In situ-assay of cyclobutane pyrimidine dimers

ARPE-19 cells were seeded onto 96-well plates at a density of 2×10^4 cells per well in growth medium. Confluent cell layers were washed and replaced with serum-free medium containing IL-1α, which were then incubated for 48 h. Direct intracellular DNA damage was measured using the in situ Cellular UV DNA Damage Detection Kit according to the manufacturer's protocol (Abnova, Taiwan) 1-3 h after UVB irradiation. Absorbance values were determined with a spectrophotometer at a wavelength of 450 nm using the reference wavelength of 655 nm.

2.9 | Western blot

Equal amounts of protein (20 μg) were run in 10% of SDS-PAGE gels and wet-blotted overnight onto nitrocellulose membranes (Amersham, Piscataway, NJ, USA). The membranes were blocked with 5% of fat-free milk in 0.1% of Tween-20/Tris-buffered saline (TBS) for 1 h at room temperature (RT). Thereafter, membranes were incubated with an anti-CIAS1/NALP3 antibody (1:1000 in 0.1% of Tween-TBS; cat. 109314, Abcam, Cambridge, UK) overnight at +4°C. On a next day, membranes were washed 3 × 5 min in 0.1% of Tween-TBS followed by a 2 h incubation at RT with a horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:5 000 in 5% of milk in 0.1% of Tween-TBS; cat. A16104, Thermo Fischer Scientific, Waltham, USA). The washing steps were repeated and the NLRP3 protein-antibody complexes were detected using the enhanced chemiluminescent assay for horseradish peroxidase (Millipore, Billerica, MA, USA) on Super Rx medical X-Ray film (Amersham HyperfilmTM ECL, GE Healthcare, Chicago, USA). The band intensities were quantified using the ImageJ software (http://rsb.info.nih.gov/ij/index.html) and normalized to α-tubulin values using protocol described previously.[19]

2.10 | Statistical analyses

All results were analyzed with GraphPad Prism Program (GraphPad Software, version 8, San Diego, CA, USA). Pairwise comparisons were performed using the Mann-Whitney *U*-test. *P*-values .05 or less were considered as statistically significant.

3 | RESULTS

3.1 | UVB induces DNA damage in RPE cells

Since cyclobutane pyrimidine dimers (CPDs) are the most abundant and cytotoxic types of DNA damage,[13,22] we exposed ARPE-19 cells to UVB and measured CPD levels at early time points (1 and 3 h) post-UVB-irradiation. UVB significantly increased the amount of DNA damage in both unprimed and IL-1α-primed cells at 1 h (mean for untreated control: 1 and UVB: 20.3, $P < .001$; mean for IL-1α: 1.1 and IL-1α + UVB: 20.5; $P < .001$; Figure 1A) or 3 h (mean for untreated control: 1 and UVB: 12.0, $P < .001$; mean for IL-1α: 0.9 and IL-1α + UVB: 12.1, $P < .001$; Figure 1A) post-UVB exposure. DNA damage was not accompanied by the rupturing of the cell membrane since IL-1α + UVB-treated cells released 2.5-fold lower amounts of LDH when compared to untreated control cells (mean for untreated control: 1 and IL-1α + UVB: 0.4, $P < .001$; Figure 1B).

3.2 | UVB induces inflammasome activation in RPE cells

In order to prime ARPE-19 cells for the inflammasome activation, cells were primed with IL-1α (4 ng/mL) for 48 h and subsequently exposed to UVB radiation. The release of IL-1β was significantly increased in IL-1α-primed ARPE-19 cells after UVB exposure when compared to cells exposed only to IL-1α or UVB (IL-1α: 0.05 pg/mL, UVB: 0.06 pg/mL, IL-1α + UVB: 0.63 pg/mL, both $P < .0001$; Figure 2A). In the absence of priming, UVB did not increase the secretion of IL-1β when compared to untreated control cells (untreated control: 0.02 pg/mL, UVB: 0.06 pg/mL, $P > .05$; Figure 2A) highlighting the importance of the priming signal for the activation of the inflammasome. Unlike IL-1β, the release of mature IL-18 was statistically significantly increased after UVB treatment both with and without priming (untreated control: 1.3 pg/mL and UVB: 519.6 pg/mL, $P < .0001$; IL-1α: 1.6 pg/mL and IL-1α + UVB: 502.8 pg/mL, $P < .0001$; Figure 2B). It is notable that the release of IL-18 was rather similar in both UVB-stimulated groups regardless of the priming signal ($P > .05$; Figure 2B) indicating that IL-18 may not necessarily require a priming step for its maturation.

After activation, caspase-1 is cleaved into p10 and p20 subunits[9]; we found that both UVB and IL-1α + UVB treatments significantly increased the levels of caspase-1 (p20 subunit) in the cell culture medium when compared to untreated or IL-1α-treated control cells, respectively (untreated control: 0.1 pg/mL and UVB: 9.5 pg/mL, $P < .0001$; IL-1α: 0.05 pg/mL and IL-1α + UVB: 18.3 pg/mL; $P < .0001$; Figure 2C). Caspase-1 levels were almost doubled in IL-1α + UVB-exposed cells when compared to the UVB-treated group ($P < .0001$; Figure 2C). Since the level of extracellular IL-1β was not significantly increased in unprimed RPE cells (Figure 2A) and the amount of caspase-1 was not at its maximum (Figure 2C) when UVB strongly induced the release of LDH (Figure 2D), it appears that the amounts of IL-1β and caspase-1 secreted from primed cells after UVB

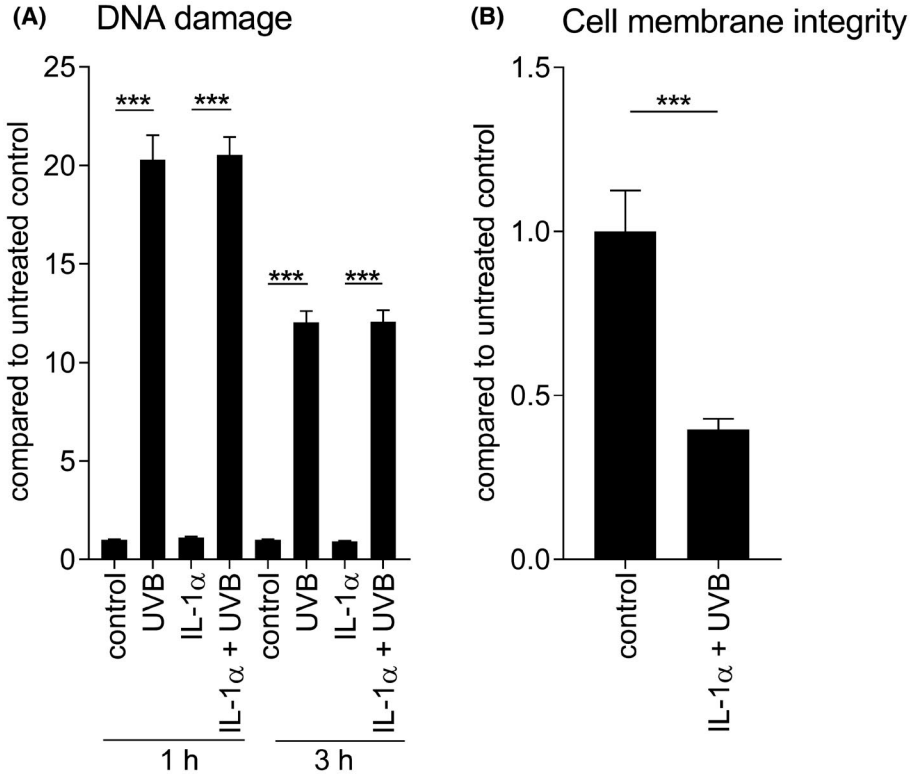

FIGURE 1 Damage induced by UVB in ARPE-19 cells. DNA damage was measured at 1 and 3 h after UVB (2.29 J/cm^2) irradiation by assaying the levels of cyclobutane pyrimidine dimers (CPDs) using a colorimetric in situ-assay (A). DNA damage was measured from two independent experiments with four to six parallel samples per group. LDH as cell membrane integrity marker was measured 3 h after UVB treatment by a commercial assay (B). LDH data were combined from three independent experiments with four parallel samples per group. Results are presented as mean ± SEM. ***$P < .001$, Mann-Whitney U-test

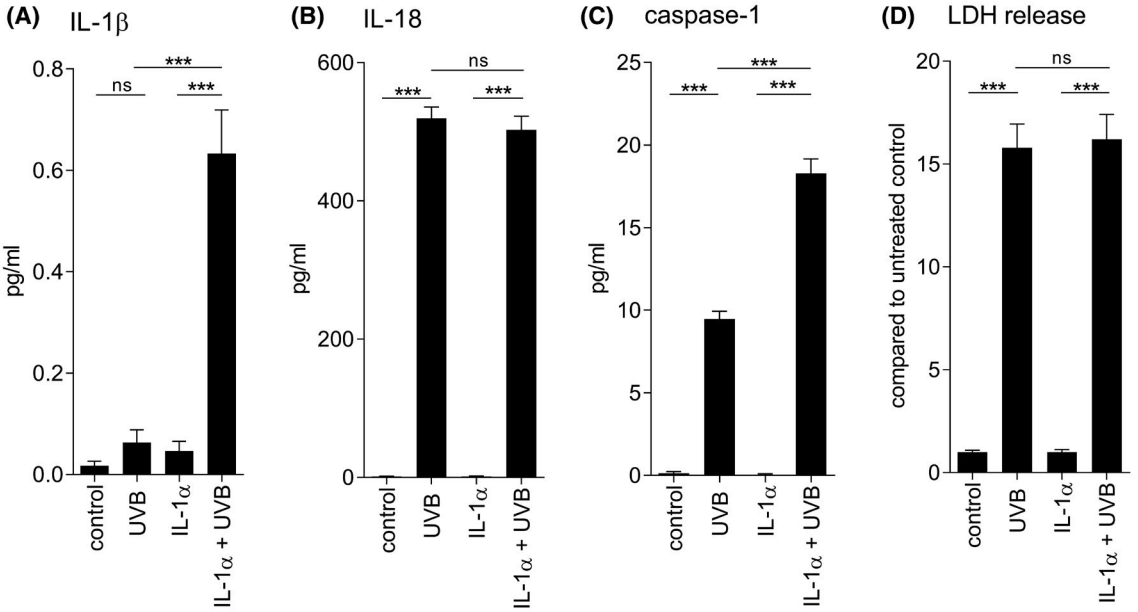

FIGURE 2 Inflammasome activation in UVB-treated ARPE-19 cells. ARPE-19 cells were primed with IL-1α (4 ng/mL) for 48 h. The released amounts of IL-1β (A), IL-18 (B), cleaved caspase-1 (C), and LDH (D) were measured at 24 h following the exposure to UVB (2.29 J/cm^2). The data have been combined from 3 to 4 independent experiments with 4-6 parallel samples per group and presented as mean ± SEM. ***$P < .0001$; ns, not significant, Mann-Whitney U-test

irradiation were being actively controlled instead of simply passively leaking due to disturbances in the integrity of the cell membrane.

3.3 | NLRP3 inflammasome contributes to the UVB-induced release of IL-1β but not to that of IL-18

After observing that IL-1α + UVB exposure induced inflammasome activation and contributed to the production of IL-1β and IL-18, next it was determined whether NLRP3 was the receptor responsible for UVB-stimulated inflammasome activation. The involvement of NLRP3 in IL-1α + UVB-induced inflammasome activation was examined by knocking down NLRP3 prior to priming with IL-1α. The protein levels of intracellular NLRP3 were reduced upon NLRP3 knockdown when compared to nonspecific siRNA ($P < .01$; Figure 3A). Thereafter, it was found that NLRP3 knockdown statistically significantly decreased the secretion of IL-1β when measured upon IL-1α + UVB exposure (nonspecific siRNA + IL-1α + UVB: 2.4 pg/mL, NLRP3 siRNA + IL-1α + UVB: 1.8 pg/mL, $P < .01$; Figure 3B). In contrast to the secretion of IL-1β, the effect of NLRP3 knockdown on the release of IL-18 was opposite since IL-1α + UVB almost doubled the levels of IL-18 (1.9 fold elevation) when compared to nonspecific siRNA control (nonspecific siRNA + IL-1α + UVB: 126.4 pg/mL, NLRP3 siRNAα + IL-1α + UVB: 239.5 pg/mL, $P < .001$; Figure 3C). These findings suggest that the release of IL-1β was dependent on and the release of IL-18 was independent of the NLRP3 inflammasome activation in DNA-damaged ARPE-19 cells.

3.4 | IL-1α priming is needed for the expression of IL-1β mRNA but not that of IL-18 in RPE cells

Since it appeared that mature IL-18 could be produced without IL-1α priming (Figure 2B), we determined whether priming evokes the expression of IL-18 and IL-1β mRNAs in RPE. Earlier studies have shown that the upregulation of pro-IL-1β is increased in a time-dependent manner and is detected at the protein level already at 3 h after the IL-1α priming.21 As expected, IL-1α-primed cells produced 16.5 and 151.9-fold higher amounts of IL-1β mRNA at both 3 h and 6 h when compared to untreated control cells, respectively (mean for untreated control: 1, IL-1α 3 h: 17.2, IL-1α 6 h: 153.8, both $P < .0001$; combined results in Figure 4A). In contrast, IL-1α did not induce the expression of IL-18 mRNA in ARPE-19 cells at 3 h post-IL-1α-treatment (mean for untreated control: 1 and IL-1α: 1.1, $P > .05$; Figure 4B) and the expression of IL-18 mRNA was even reduced at 6 h after IL-1α treatment when compared to the basic level of the untreated

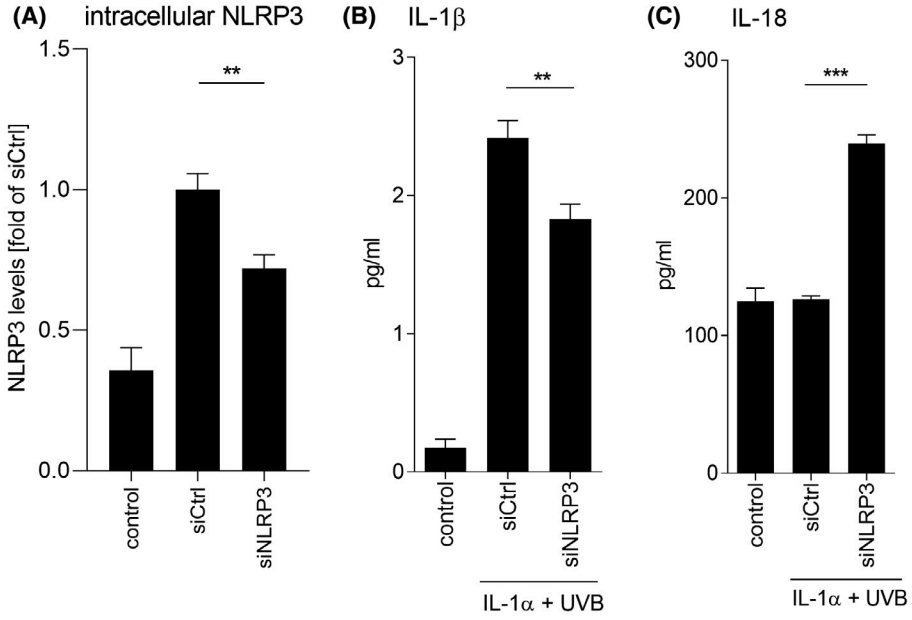

FIGURE 3 The role of NLRP3 on the UVB-induced inflammasome activation in ARPE-19 cells. NLRP3 knockdown was performed 24 h prior to IL-1α priming (4 ng/mL). Thereafter, at 24 h after priming, ARPE-19 cells were irradiated under the UVB light (2.29 J/cm^2). Prior to IL-1α priming, intracellular protein levels of NLRP3 were measured by ELISA and the results were normalized to total protein concentrations (A). The secreted amounts of IL-1β (B) and IL-18 (C) were measured 24 h after UVB exposure using the ELISA method. The data have been combined from two (A-C) independent experiments with three to four (A) or six (B-C) parallel samples per group. Results are presented as mean ± SEM. $^{**}P < .01$, $^{***}P < .001$, Mann-Whitney *U*-test. siCtrl = nonspecific siRNA, siNLRP3 = NLRP3 siRNA

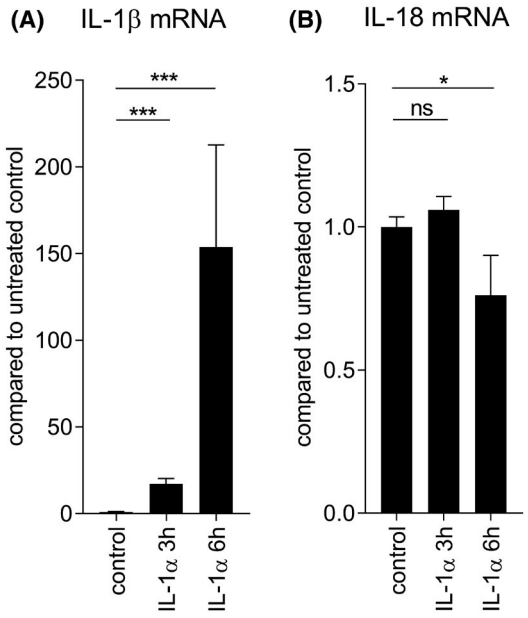

FIGURE 4 Expression of IL-1β and IL-18 mRNAs following the IL-1α priming of ARPE-19 cells. IL-1β (A) and IL-18 (B) mRNA levels were detected 3 h and 6 h after the IL-1α priming (4 ng/mL) by quantitative RT-PCR. Data were combined from two independent experiments with four to six parallel samples per group. qRT-PCR samples were run in duplicate. Results are shown mean ± SEM. *$P < .05$, ***$P < .001$; ns, not significant, Mann-Whitney *U*-test

control (mean for untreated control: 1 and IL-1α: 0.8, $P < .05$; Figure 4B). This suggests that the IL-1α-treated cells induced the expression of pro-IL-1β but not that of pro-IL-18.

3.5 | Production of IL-18 is regulated by ROS in UVB-irradiated RPE cells

In order to clarify the mechanisms behind the UVB-regulated production of IL-1β and IL-18, ROS activity was measured from the cells using the cell-permeant fluorogenic dye, H_2DCFDA. The production of ROS was initiated immediately after UVB exposure in unprimed and IL-1α-primed RPE cells (Mean RFU for untreated control: 2233.7 and UVB: 14 546.5, $P < .001$; mean RFU for IL-1α: 2636.7 and IL-1α + UVB: 14 993.3, $P < .001$; Figure 5A). The glutathione precursor, n-acetyl-cysteine (NAC) at concentrations of 5 and 10 mM significantly reduced ROS levels in primed RPE cells exposed to UVB (mean RFU for IL-1α + UVB: 14 993.3, IL-1α + NAC 10 mM + UVB: 9704.8 and IL-1α + NAC 5 mM + UVB: 11 380.3, both $P < .001$; Figure 5A). Subsequently, the release of IL-18 was decreased by 5 mM of NAC (mean for IL-1α + UVB: 1 and IL-1α + NAC 5 mM + UVB: 0.8, $P < .01$; Figure 5B). In contrast, NAC significantly increased the release of IL-1β (mean for IL-1α + UVB: 1 and IL-1α + NAC 5 mM + UVB: 2.4, $P < .001$; Figure 5B). The responses were similar when the cells were pretreated with another ROS scavenger, MitoTEMPO, which significantly reduced the secretion of IL-18 (mean for IL-1α + UVB: 1 and IL-1α + MitoTEMPO 200 μM + UVB: 0.7, $P < .001$; Figure 5C). In line with the results shown in Figure 5B, MitoTEMPO more than doubled the release of IL-1β (2.2 fold elevation) when compared to cells without MitoTEMPO pretreatment (mean for IL-1α + UVB: 1 and IL-1α + MitoTEMPO 200 μM + UVB: 2.2, $P > .05$; Figure 5C).

3.6 | The release of IL-1β is regulated by potassium efflux in UVB-exposed RPE cells

Since ROS production appeared to be associated with the release of IL-18 but not that of IL-1β in UVB-exposed ARPE-19 cells, we continued to clarify the mechanisms by exploring the potential role of potassium efflux, as this has been claimed to be one of the major activation mechanisms of the NLRP3 inflammasome.[23,24] In order to prevent K^+ efflux, ARPE-19 cells were exposed to 50 mM of extracellular KCl prior to UVB exposure. This treatment resulted in a statistically significant reduction in IL-1β release from the primed RPE cells stressed with UVB (mean for IL-1α + UVB: 1 and IL-1α + KCl +UVB: 0.4, $P < .001$; Figure 6A). Concurrently, extracellular KCl statistically significantly increased the release of IL-18 (mean for IL-1α + UVB: 1 and IL-1α + KCl +UVB: 1.1, $P < .001$; Figure 6A). Excess levels of extracellular KCl also reduced ROS activity in untreated and IL-1α-primed cells (mean RFU for untreated control: 3593.4 and KCl: 2138.2, $P < .001$; mean RFU for IL-1α: 4384.6 and IL-1α + KCl: 2254.5, $P < .001$; Figure 6B) suggesting that K^+ efflux plays a role in the priming phase. However, high extracellular potassium neither decreased nor increased the intracellular NLRP3 levels (Figure S1). Unlike the elevated ROS levels can promote a pathway upstream of NLRP3 induction,25 our findings propose that K^+ efflux alone does not directly regulate the expression of NLRP3. It appears that IL-1α-induced K^+ efflux associated ROS production can stimulate the priming phase in RPE cells as shown previously.[17] Instead, blockade of the K^+ efflux by excessive extracellular KCl rather stimulated than mitigated ROS production upon UVB exposure (mean RFU for IL-1α + UVB: 21 892.6 and IL-1α + KCl +UVB: 27 175.8, $P < .001$; Figure 6B) demonstrating the different role of KCl in the priming vs. activation stage of NLRP3 inflammasome in ARPE-19 cells.

Since extracellular ATP was able to induce K^+ efflux resulting in NLRP3 activation,[26] finally, we explored whether UVB could induce the release of ATP from ARPE-19 cells. ATP was actively released at 3 h post-UVB-treatment, and the ATP levels were 3.9-fold higher as compared to the

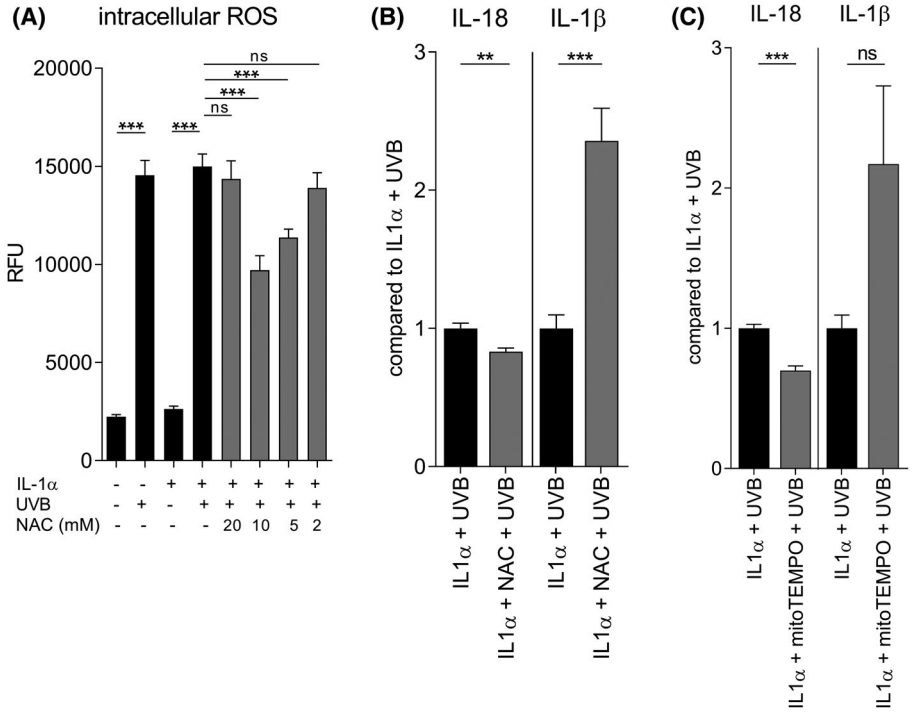

FIGURE 5 The role of ROS in UVB-induced inflammasome activation in ARPE-19 cells. IL-1α-primed (4 ng/mL for 48 h) ARPE-19 cells were irradiated with UVB (2.29 J/cm^2). ROS production was measured by the DCFDA assay immediately after UVB exposure with or without NAC pretreatment (A). The effects of NAC (B) and MitoTEMPO (C) on the release of IL-18 and IL-1β were evaluated 24 h after UVB exposure by ELISA. DCFDA data were combined from three independent experiments with eight parallel samples per group, whereas IL-18 and IL-1β data were combined from two independent experiments with four to six parallel samples in each group. Results are shown as mean ± SEM. $^{**}P < .01$, $^{***}P < .001$; ns, not significant, Mann-Whitney *U*-test

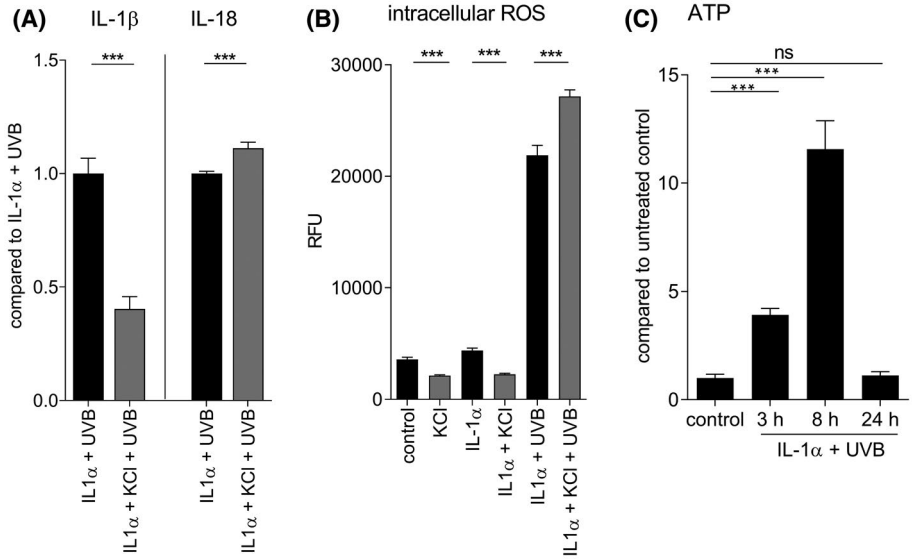

FIGURE 6 The effect of high extracellular potassium on the UVB-stimulated inflammasome activation in ARPE-19 cells. ARPE-19 cells were primed with IL-1α (4 ng/mL) and cultured with excessive amount of KCl (50 mM) for 48 h prior to UVB exposure (2.29 J/cm^2). Levels of IL-1β and IL-18 were measured 24 h after UVB stimulation from cell culture medium samples by an ELISA method (A). Intracellular amounts of ROS with the DCFDA assay were performed immediately after UVB irradiation (B). ATP release was determined by a luciferase-based bioluminescence assay (C). Data were combined from four (IL-1β) or two (IL-18 and DCFDA) independent experiments with 4-6 (IL-1β), 5 (IL-18), or 8 and 15 (DFCDA) parallel samples in each group. ATP levels were measured from two (3 h and 8 h time points) or three (24 h time point) independent experiments with four samples per group. Results are shown as mean ± SEM. $^{***}P < .001$; ns, not significant, Mann-Whitney *U*-test

untreated control cells ($P < .001$; Figure 6C). Extracellular ATP levels were further increased with time, ie at the 8 h time point the level was 11.6-fold higher when compared to untreated control cells ($P < .001$; Figure 6C). ATP concentration returned to control levels at 24 h after the exposure to UVB (mean for untreated control: 1 and IL-1α + UVB: 1.1, $P > .05$; Figure 6C).

4 | DISCUSSION

The probability of DNA damage increases during aging due to increased levels of cellular stress and the reduced capacity to repair damaged macromolecules. The exact mechanisms behind many age-dependent diseases are still unknown but the contribution of DNA-related stress cannot be excluded. Inflammation is the principal response to any factor threatening cellular homeostasis, and inflammasome signaling has been one of the most recent pathways to be identified in the induction of inflammation.[27] Inflammasome activation has been associated with several chronic diseases,[28,29] including AMD.[27] For example, the end product of lipid peroxidation, 4-hydroxynonenal,[30] defective protein clearance,[31] blue light irradiation in conjunction with lipofuscin accumulation,[32] oxidative stress induced by lipopolysaccharide (LPS), and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD),[33] lysosomal destabilization,21 and the accumulation of *Alu* RNA[34,35] are capable of activating inflammasome signaling in human RPE cells. However, it is still unknown whether inflammasome activation is involved in DNA damage in the human RPE.

The present study demonstrated that inflammasomes can be activated by UVB irradiation in RPE cells. Previously, UVB has been shown to induce the production of IL-1β in human keratinocytes,[36,37] where NLRP3 was recognized to be the responsible inflammasome receptor.[37] Our present data are in line with the previous studies on keratinocytes,[36,37] showing that UVB-induced production of IL-1β is a result of NLRP3 activation. Although two signals are typically needed for the activation of the NLRP3 inflammasome, keratinocytes appear to manage without priming.[36,37] In our RPE cells, priming was necessary to evoke the UVB-induced IL-1β maturation but dispensable for the UVB-stimulated maturation of IL-18. Previously, Shi et al[20] reported that pro-IL-18 can be constitutively expressed in human RPE cells, which is in line with the findings of our study. The same phenomenon has been observed previously, for example, in studies performed with corneal epithelial cells, peripheral blood mononuclear cells, keratinocytes, endothelial cells, and epithelial cells of the gastrointestinal tract.[5,38,39]

Similarly to priming, the release of mature IL-18 was not dependent on NLRP3 inflammasome activation. This is the first study that has investigated the distinct mechanisms for the secretion of IL-1β and IL-18 in retinal cells. Moreover, our study is in line with Hanamsagar et al,[40] who previously found that exposure of human microglia to *Staphylococcus aureus* resulted in NLRP3-mediated release of IL-1β but in contrast, IL-18 secretion was independent of NLRP3. Additionally, it has been reported that the cleavage of pro-IL-18 can occur independently of inflammasome activation, for example, epithelial cell-derived metalloproteinase meprin β is capable of processing IL-18.[41] Moreover, activated caspase-8 was able to splice the pro-form of IL-18 without canonical inflammasome activation.[42,43] Although the activation mechanism of IL-18 in RPE cells still needs to be clarified, a role for bioactive IL-18 has been proposed in some ocular diseases. Recently, an increased level of IL-18 has been detected in human proliferative diabetic retinopathy.[44] Moreover, accumulated *Alu* RNA[35,45] and blue light[32] have been shown to induce the secretion of IL-18 from RPE cells, pointing to a role for this cytokine in AMD. In conjunction with the secretion of IL-18, UVB without the priming signal was capable of inducing the activation of caspase-1 although the levels were significantly higher in primed ARPE-19 cells. Unprimed and caspase-1-activated ARPE-19 cells did not seem to secrete IL-1β after UVB exposure. Previously, similar UVB-induced caspase-1 activation has been reported by Sollberger et al[46] who demonstrated that in addition to inflammasome activation, UVB could induce inflammasome-independent but caspase-1-dependent apoptosis in human keratinocytes.

As prominent IL-18 release resulted from UVB exposure, this was closely correlated with DNA damage and ROS production. This is in line with studies suggesting that the production of IL-18 contributes to the repair response triggered in response to UV-induced DNA damage through the activation of the nucleotide excision repair (NER) system and the prevention of apoptosis.[47] It is also known that in addition to DNA, UVB-induced ROS can attack other cellular targets, for example, some proteins are specially vulnerable to both direct oxidation and covalent binding of lipid peroxidation end products.[48] Therefore, our present data demonstrating IL-18 release from ARPE-19 cells is also in line with our previous study, in which 4-hydroxynonenal promoted the release of IL-18 but not of IL-1β from RPE cells.[30] These findings are in agreement with Cho et al who found that ROS were needed for IL-18 production in the human keratinocyte (HaCaT) cell line.[49]

K^+ efflux has been associated with the NLRP3 inflammasome-related IL-1β release in various studies,23-24,26,50 and our present data support this observation. We found that K^+ efflux was involved in the secretion of IL-1β in UVB-irradiated RPE cells. It appears that ROS do not have a role in the processing of IL-1β, which is in line with studies conducted with linezolid-stimulated macrophages[50] and primary monocytes from patients with a granulomatous disease,[51] ie the secretion of IL-1β is independent of ROS. With regard

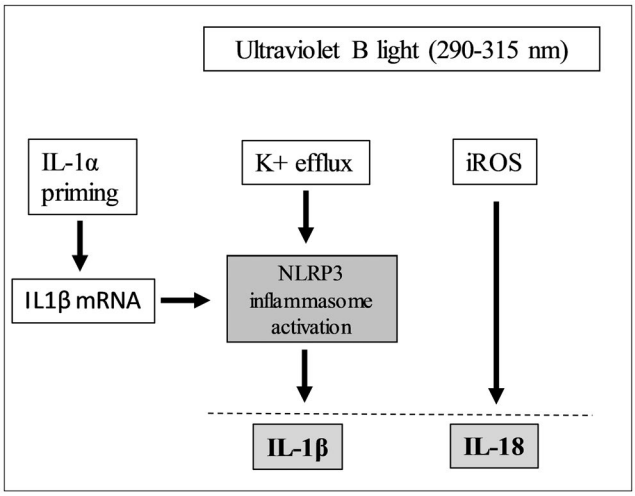

FIGURE 7 Summary presentation of the main findings of this study. IL-1α induced the expression of IL-1β mRNA but not that of IL-18. UVB stimulated the NLRP3 inflammasome-dependent secretion of IL-1β but the release of IL-18 was independent of NLRP3 inflammasome activation. K^+ efflux was associated with the secretion of IL-1β, whereas intracellular ROS (iROS) production was an upstream event prior to the secretion of IL-18

to our present finding, it is interesting to note that UVB has previously been shown to activate K^+ channels[52] via tumor necrosis factor receptor 1[53] in human corneal epithelial cells. This suggests that there is a link between UVB induced K^+ channel activation and the regulation of IL-1β. Moreover, ATP has been shown to play a role in K^+ efflux and Ca^{2+} influx-mediated NLRP3 inflammasome activation in human and murine neutrophils.[26] Additionally, extracellular ATP has been proposed to regulate NLRP3 inflammasome activation in human macrophages.[54,55] In agreement with the previous findings, our present data indicate that ATP may trigger an early cellular event leading to ion fluctuations in K^+ efflux-mediated NLRP3 activation since extracellular levels of ATP were time-dependently increased.

In conclusion, we have shown here for the first time that UVB-induced DNA damage disturbed the normal homeostasis of RPE cells, and this resulted in inflammasome activation. Inflammasome-regulated cytokines are typically grouped together leading to the impression that inflammasome and caspase-1 activation automatically cleaves both pro-forms of IL-1β and IL-18 into their mature forms. Our present study highlights the fact that even in the same cell, the same activator can exert distinct effects. We have reported here that DNA damage and ROS production contributed to the maturation of IL-18, whereas IL-1β release was dependent on K^+ efflux-mediated NLRP3 inflammasome activation (Figure 7). These findings provide new approaches to understand mechanisms behind the chronic eye disease, AMD. Due to disease complexity, 80% of AMD patients suffer from the dry form to which there is no approved treatment available at the moment.[56] Additionally, our observation that inflammasome-related cytokines IL-1β and IL-18 have distinct signaling pathways upon same activator affords novel information to understand better the inflammasome signaling also in other than RPE cells. Therefore, our findings can be utilized in research of various chronic diseases, where inflammasomes are involved in the disease progression, for example, Alzheimer's disease and Parkinson's disease.[57-59]

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

E. Korhonen and A. Kauppinen designed the study; E. Korhonen, N. Piippo, and M. Hytti performed the experiments; J. M. T. Hyttinen contributed new reagents or analytic tools; E. Korhonen analyzed the data; A. Kauppinen and K. Kaarniranta contributed to materials and analysis tools; E. Korhonen and A. Kauppinen wrote the paper, and others critically reviewed, commented, and provided their suggestions to the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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